

The Listing of Claims

1. (Currently Amended) A methylotrophic recombinant yeast strain for producing a human insulin precursor, the strain having a genome comprising a copy of a first DNA construction and a copy of a second DNA construction, wherein said constructions controlling are capable of directing the expression and secretion of a human insulin precursor, and wherein said DNA constructions comprisingcomprise at least one DNA sequence encoding a human insulin precursor and analogousanalogs thereof.

2. (Currently Amended) The yeast strain of claim 1, wherein the strain secretedsecretes a human insulin precursor of the formula:

B(1-30)-Y1-Y2-A(1-21), wherein Y1 is lysine or arginine; Y2 is lysine or arginine; B(1-30) is the B peptide of the human insulin; and B(1-21) is the A peptide of human insulin.

3. (Currently Amended) The yeast strain of claim 1, wherein the strain is a member selected from *Hansenula*, *Pichia*, *Candida*, and *Torulopsis*.

4. (Currently Amended) The yeast strain of claim 3, wherein the strain is yeast *Pichia* *Pastoris**pastoris* deposited under accession number ATCC PTA-2260.

5. (Currently Amended) The yeast strain of claim 1, wherein the first DNA construction comprises:

a1) a first insertableinsertion DNA sequence corresponding to a 5' regulatory region (promoter) operably linked to

b1) an exporting signal sequence operableoperably linked to

c1) a sequence encoding a human insulin precursor operableoperably linked to

d1) a 3' termination sequence linked to

e1) a selectable gene linked to

f1) a second ~~insertable~~insertion DNA sequence; and
the second DNA construction comprises:
a2) a first ~~insertable~~insertion DNA sequence corresponding to a 5' regulatory region (promoter) operably linked to
b2) an ~~exporting~~ signal sequence ~~operable~~operably linked to
c2) a sequence encoding a human insulin precursor ~~operable~~operably linked to
d2) a 3' termination sequence linked to
e2) a selectable gene ~~distinct~~different from the selectable gene of the first DNA construction.

6. (Withdrawn - Currently Amended) The first DNA construction of claim 5, comprising at least one expression cassette for expressing the human insulin precursor, ~~thewherein said~~ cassette ~~comprising~~comprises:

- a) a 5' regulatory region operably linked to
- b) a DNA sequence encoding a signal sequence ~~operable~~operably linked to
- c) a sequence encoding a human insulin precursor ~~operable~~operably linked to
- d) a functional transcription termination sequence.

7. (Withdrawn) The first DNA construction of claim 5, wherein the selectable gene is HIS4.

8. (Withdrawn - Currently Amended) The first DNA construction of claim 5, wherein the 5' and 3' ends of said DNA construction comprises sequences ~~enough~~which are sufficiently homologous ~~with~~to a target gene of the yeast to ~~permit~~allow for the replacement by a specific insertion of the DNA construction in ~~the~~a target gene, in the same relative orientation of the target gene in the yeast genome.

9. (Withdrawn) The first DNA construction of claim 5, wherein the 5' regulatory region is selected from the group consisting of the *Pichia pastoris* primary alcohol oxidase enzyme (AOX1) gene promoter, the secondary alcohol oxidase II enzyme (AOX2) gene promoter, the *Pichia pastoris* dihydroxyacetone synthase (DAS) gene promoter, the *Pichia pastoris* p40 regulatory regions promoter, the *Pichia pastoris* catalase promoter, and the glyceraldehyde dehydrogenase GAP promoter.

10. (Withdrawn) The first DNA construction of claim 5, wherein the signal sequence is the MF α of *Saccharomyces cerevisiae*.

11. (Withdrawn - Currently Amended) The first DNA construction of claim 5, wherein the functional transcription termination sequence is the termination sequence derived from *Pichia pastoris* AOX1 gene.

12. (Withdrawn - Currently Amended) The first DNA construction of claim 5, further comprising:

- a) a first insertableinsertion DNA sequence corresponding to a 5' regulatory region of *Pichia pastoris* AOX1 gene operably linked to
- b) the MF α signal sequence of *Saccharomyces cerevisiae* operableoperably linked to
- c) the sequence encoding the human insulin precursor of formula B(1-30)-Y1-Y2-A(1-21) operableoperably linked to
 - d) a 3' termination sequence of *Pichia pastoris* AOX1 gene operably linked to
 - e) a *Pichia pastoris* HIS4 selection gene operably linked to
 - f) a second insertableinsertion DNA sequence corresponding to the *Pichia pastoris* AOX1 gene termination sequence.

13. (Withdrawn - Currently Amended) The first DNA construction of claim 5, wherein the sequence encoding the human insulin precursor is cloned in said construction

~~following~~adjacent to the protease site of the signal peptide, wherein all the secreted human insulin ~~precursor~~ contains, in its amino terminal end, ~~precursors~~ contain the first amino acid of the human insulin precursor in the amino terminal end.

14. (Withdrawn) The first DNA construction of claims 5-13, wherein said DNA construction is incorporated into a vector selected from the group consisting of linear and circular vectors.

15. (Withdrawn - Currently Amended) The second DNA construction of claim 5, comprising at least one expression cassette for expressing the human insulin precursor, ~~th~~wherein said cassette comprisingcomprises:

- a) a 5' regulatory region operably linked to
- b) a DNA sequence encoding a signal sequence ~~operable~~operably linked to
- c) a sequence encoding a human insulin precursor ~~operable~~operably linked to
- d) a functional transcription termination sequence.

16. (Withdrawn - Currently Amended) The second DNA construction of claim 5, further comprising a selectable gene distinct from the selectable gene of the first DNA construction, wherein said selectable gene is the gene encoding zeocine resistance and wherein said selectable gene ~~permits to carry out~~allows for a second selection event.

17. (Withdrawn - Currently Amended) The second DNA construction of claim 5, wherein the 5' end of said insertion DNA construction comprises a single sequence ~~enough~~sufficiently homologous with to a target gene of the yeast to permitallow for the integration of the DNA construction in the target gene, in a single event.

18. (Withdrawn) The second DNA construction of claim 5, wherein the 5' regulatory region is selected from the group consisting of the *Pichia pastoris* primary alcohol

oxidase enzyme (AOX1) gene promoter, the secondary alcohol oxidase II enzyme (AOX2) gene promoter, the *Pichia pastoris* dihydroxyacetone synthase (DAS) gene promoter, the *Pichia pastoris* p40 regulatory regions promoter, the *Pichia pastoris* catalase promoter, and the glyceraldehyde dehydrogenate GAP promoter.

19. (Withdrawn) The second DNA construction of claim 5, wherein the signal sequence is MF α *Sacharomyces cerevisiae*.

20. (Withdrawn - Currently Amended) The second DNA construction of claim 5, wherein the functional transcription termination sequence is the termination sequence derived from the *Pichia pastoris* AOX1 gene.

21. (Withdrawn - Currently Amended) The second DNA construction of claim 5, further comprising:

- a) a first insertableinsertion DNA sequence corresponding to a 5' regulatory region of the *Pichia pastoris* AOX1 gene operably linked to
- b) the MF α signal sequence of *Sacharomyces cerevisiae* operableoperably linked to
- c) thea sequence encoding the human insulin precursor of formula B(1-30)-Y1-Y2-A(1-21) operableoperably linked to
- d) a 3' termination sequence of the *Pichia pastoris* AOX1 gene operably linked to
- e) thea zeocine-resistant selection gene.

22. (Withdrawn - Currently Amended) The second DNA construction of claim 5, wherein the sequence encoding the human insulin precursor is cloned in said construction followingadjacent to the protease site, wherein all the secreted human insulin precursor contains, in its amino terminal end, precursors contain the first amino acid of the human insulin precursor in the amino terminal end.

23. (Withdrawn) The second DNA construction of claims 15-22, wherein said DNA construction is incorporated into a vector selected from the group consisting of linear and circular vectors.

24. (Currently Amended) A method ~~of~~ for obtaining the yeast strain of claim 1, comprising the steps of:

i) transforming a yeast cell with a first DNA construction, ~~comprising: wherein said construction comprises:~~

a) a first ~~insertable~~insertion DNA sequence corresponding to a 5' regulatory region of the Pichia pastoris AOX1 gene operably linked to

b) the MF α signal sequence of Sacharomyces cerevisiae operably linked to

c) ~~the-a~~ sequence encoding the human insulin precursor of formula B(1-30)-Y1-Y2-A(1-21) ~~operable~~operably linked to

d) a 3' transcription termination sequence of the Pichia pastoris AOX1 gene operably linked to

e) a Pichia pastoris HIS4 selection gene operably linked to

f) a second ~~insertable~~insertion DNA sequence corresponding to the Pichia pastoris AOX1 gene termination sequence;

ii) selecting the yeast cells;

iii) isolating a yeast strain;

iv) re-transforming the yeast strain obtained in steps i)-iii) with a second DNA construction, ~~comprising: wherein said construction comprises:~~

a) a first ~~insertable~~insertion DNA sequence corresponding to a 5' regulatory region of the Pichia pastoris AOX1 gene operably linked to

b) the MF α signal sequence of Sacharomyces cerevisiae operably linked to

c) the sequence encoding the human insulin precursor of formula B(1-30)-Y1-Y2-A(1-21) ~~operable~~operably linked to

d) a 3' transcription termination sequence of the *Pichia pastoris* AOX1 gene operably linked to

- e) the a zeocine-resistant selection gene;
- v) selecting the re-transformed yeast strain; and
- vi) isolating the selected and re-transformed yeast strain.